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Asplenium ceterach and *A. octoploideum* on the Canary Islands (Aspleniaceae, Pteridophyta)

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ABSTRACT.—Isozyme and plastid DNA analysis prove that true *A. ceterach* occurs on the Canary Islands, in addition to *A. aureum* and an octoploid taxon. Combining morphological and cytological observations leads to correct determination, but the exospore length alone also allows reliable identification of these Canarian species. Our allozyme data suggest that the Canarian *A. ceterach* population is not completely genetically isolated from the European ones. The holotype of *Ceterach aureum* var. *parvifolium*, formerly regarded as an octoploid taxon, proved to be *A. ceterach*, leaving the octoploid without a correct name. The recently described *A. octoploideum* shows monomorphic, presumably fixed heterozygosity for a combination of the patterns seen in *A. ceterach* and *A. aureum* at four loci (*Aat, Skdh, Me*, and *Pgi-2*) confirming its allo-octoploid nature. It most probably originated by chromosome doubling in a tetraploid hybrid between *A. aureum* and *A. ceterach* or via the union of their unreduced gametes. *Pgi-2* indicates multiple origins of the allo-octoploid, implicating recurrent gene flow from tetraploids to octoploids.

Asplenium subgenus *Ceterach* (Willd.) Bir *et al.* is a small group of about nine fern taxa within the large (720 species), subcosmopolitan genus *Asplenium* L. (Kramer and Viane, 1990). This subgenus contains xerophytic rock ferns with the dorsal side of the lamina densely covered with reddish-brown scales (=paleae). Van den heede *et al.* (2003) have shown that the group must be restricted to the Eurasian and Macaronesian species.

Ever since the description of *Asplenium aureum* Cav. from Tenerife by Cavanilles (1801), it has been unclear how many "*Ceterach*" species are extant in the Canarian Archipelago, and whether the "European" *A. ceterach* L. (Syn.: *Ceterach officinarum* Willd.) occurs in Macaronesia (Table 1). This confusion was caused by the lack of distinctive characters to distinguish both species. Cavanilles (1801) and Bory de St. Vincent (1802) mentioned only the much larger size of *A. aureum* compared to that of *A. ceterach*. Willdenow (1810) introduced the concept of "toothed scales" as a diagnostic feature, whereas Milde (1865) claimed that "Cuticularstreifen" (cuticular lines or ridges on the periclinal cell walls of the scales) could be used to distinguish *A. aureum* from

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TABLE 1. Taxa covered by names found in the literature. Abbreviations used: *A. cet.* = *A. ceterach*, *A. aur.* = *A. aureum*, *A. lol.* = *A. lolegnamense*, *A. par.* = "*A. parvifolium sensu Vida and Reichst.*" = *A. octoploideum*. ■: the filled symbol indicates that the taxon was included in this author's concept of the species mentioned in column 2; □: symbol indicates that this taxon was included in the author's concept of the species mentioned in column 2 prior to its formal description.

		Taxa included in this name			
Literature reference	Name used or published	*A. cet.*	*A. aur.*	*A. lol.*	"*A. par.*"
Linnaeus 1753	*A. ceterach*	■			
Cavanilles 1801	*A. aureum*		■		
Bory de St. Vincent 1802	*A. ceterach*	■			
	A. latifolium		■		
Desvaux 1827	*C. aurea*		■		
von Buch 1828 ["1825"]*	*C. aureum*		■		
Moore 1857	*C. aureum*		■		
	C. officinarum	■			
Lowe, E. J. 1858	*C. officinarum*	■		□	
Hooker 1860, 1861	*A. ceterach*	■			
	A. ceterach var. *aureum*		■	□	
Bolle 1864	*C. aureum*		■		
	C. officinarum	■		□	□
Milde 1866b, 1867a,b	*C. aureum*		■	□	
	C. officinarum	■		□	□
Kuhn 1868	*C. aureum*		■	□	
Sauer 1880	*C. aureum*			□	
	C. officinarum	■			
Luerssen 1889	*C. officinarum*	■	■	□	□
Schneider 1892	*A. ceterach*	■			
	A. ceterach var. *aureum*		■	□	
Christ 1897	*C. aureum*		■		
	C. officinarum	■		□	
Burchard 1929	*C. aureum*		■		
	C. officinarum	■			□
Chevalier 1935	*C. officinarum*	■			
	C. officinarum var. *aureum*		■	□	
Tardieu-Blot 1946	*C. officinarum*	■	■	□	
Copeland 1947	*C. aureum*		■		□
	C. officinarum	■			
Manton 1950	*C. aureum*		■		
	C. officinarum	■			
Romariz 1953	*C. aureum*		■	□	
Lems 1958, 1960	*C. aureum*		■	□	
	C. officinarum	■			□
Dansereau 1961	*C. aureum*	■	■	□	
Fabbri 1965	*C. aureum*		■		
	C. officianarum	■			
Kunkel 1965	*C. aureum*		■	□	□
Benl and Kunkel 1967	*C. aureum* var. *aureum*		■		
	C. aureum var. *parvifolium*	■			■
Lid 1967	*C. aureum*	■	■		
Hansen 1969	*C. aureum*			□	
Benl and Sventenius 1970	*C. aureum* var. *aureum*		■		
	C. aureum var. *parvifolium*	■			■

TABLE 1. Continued.

Literature reference	Name used or published	Taxa included in this name			
		A. cet.	*A. aur.*	*A. lol.*	*"A. par."*
Kunkel 1971	*C. aureum* var. *aureum*		■		
	C. aureum var. *parvifolium*	■			■
Hansen and Sunding 1979	*C. aureum* var. *aureum*		■	□	
	C. aureum var. *parvifolium*	■			■
Reichstein 1984	*C. aureum* var. *aureum*		■		
	C. aureum var. *parvifolium*				■
	C. offinarum	■			
Bir et al. 1985	*A. aureum*		■	□	■
	A. ceterach ssp. *ceterach*	■			
Manton et al. 1986	*C. aureum* var. *aureum*		■		
	C. aureum var. *parvifolium*				■
Gibby and Lovis 1989	*C. lolegnamense*			■	
Ormonde 1990	*C. aureum* var. *aureum*		■		
	C. aureum var. *madeirense*			■	
	C. aureum var. *parvifolium*	■			■
Viane and Reichstein 1992	*A. parvifolium*				■
	A. lolegnamense			■	
Griffiths 1997	*A. aureum*		■	■	
	A. ceterach	■			
Hoshizaki and Moran 2001	*C. aureum*		■	■	■
	C. officinarum	■			

* According to Stafleu and Cowan (1976) this book was only published after 28 May 1828, it is not clear whether Link or von Buch made the combination "*C. aureum*", which is, in any case, antedated by Desvaux (1827).

its continental counterpart, though he soon (Milde 1866a, 1866b, 1867a, 1867b) cast doubt on the utility of this character. As early as May 1866, Milde admitted that "die Cuticularstreifen, welche die Spreuschuppen von *C. aureum* stets zeigen, fand ich nun auch an exemplaren, die sich von *C. officinarum* nicht unterscheiden liessen." Finally, he came to the conclusion that the character was useless to discriminate *A. ceterach* from *A. aureum* (Milde, 1867b). Nevertheless, Bornmüller (Plantae exsicc. Canarienses–1901), and Benl and Kunkel (1967) heavily relied on this character to recognize taxa. Since 1970, chromosome numbers were used to distinguish species in this group (T. Reichstein, pers. comm.), and morphological characters became less important (e.g., Bir *et al.*, 1985; Manton *et al.*, 1986; Gibby and Lovis, 1989).

In 1967, Benl and Kunkel considered all Canarian plants that looked like *A. ceterach* to be a dwarfed variety of *A. aureum*. Unfortunately, their variety *Ceterach aureum* (Cav.) Desv. var. *parvifolium* Benl and G.Kunkel was published without cytological information. In March 1967, T. Reichstein collected living "*A. ceterach*" on Gran Canaria, and sent material for chromosome counts to G. Vida. In 1970, these plants were found to be octoploid, but because good cytological photographs were lacking the results were not published (T. Reichstein, pers. comm.). From then onwards, but without studying the type of *A. parvifolium* (Benl and G.Kunkel) Vida and

Reichst., octoploid status was attributed to it. To clarify the origin of *A. parvifolium*, a hybridization program was started by G. Vida in Budapest; results were partly published in Manton *et al.* (1986). Meanwhile, T. Reichstein had informed many pteridologists about the putative allo-octoploid nature of *A. parvifolium* and briefly mentioned it in Hegi (1984).

To date two cytologically different endemic species are generally accepted to occur on the Canary Islands: *A. aureum* and *A. parvifolium*. *Asplenium aureum* was found to be tetraploid by Manton (1950). Vida and Reichstein (Vida, 1972; Viane and Reichstein, 1992) suggested *A. aureum* to be allotetraploid, which was confirmed by ITS analysis (Van den heede *et al.*, 2003). The name *A. parvifolium* was used for the allo-octoploid that probably formed by chromosome doubling of the tetraploid hybrid between *A. aureum* and *A. ceterach* (Vida, 1972; Viane and Reichstein, 1992). After 1970, all small Canarian plants that looked like *A. ceterach* were considered to be a) *A. parvifolium* and b) octoploid. According to Manton *et al.* (1986) "*C. officinarum* is not positively recorded from Macaronesia, but its former presence, at least in the Canaries, is suggested by the morphology of some representatives of *C. aureum sens. lat.* from these islands."

Within *A. ceterach sensu lato* three cytotypes are known, and according to the Biological Species Concept (Mayr, 1942, 2000; see review in King, 1993), autopolyploids should be considered separate species because they produce sterile hybrids with their parents from which they are reproductively isolated. Diploid *A. javorkeanum* Vida [Syn.: *A. ceterach* ssp. *bivalens* (D.E. Mey.) Greuter and Burdet; *C. officinarum* Willd. ssp. *bivalens* D.E. Mey.] is known from Albania, Bulgaria, Croatia, Greece, Hungary, Italy, Romania, and Slovenia (Vida, 1963; Reichstein, 1984), and should be looked for in northern Algeria and Turkey, because the triploid hybrid *A.* ×*mantoniae* Váróczy and Vida was found there (Greuter, 1980; Viane *et al.*, 1996). Tetraploid (Manton, 1950; Vida, 1963) *A. ceterach* [Syn.: *C. officinarum* Willd. ssp. *officinarum*] is supposed to have originated via chromosome doubling in *A. javorkeanum*; its autopolyploid status was confirmed cytologically by Rasbach *et al.* (1987). The autotetraploid is the more common species, occurring throughout Europe (see maps in Jalas and Suominen, 1972; Pichi Sermolli, 1979; Reichstein, 1984), southwestern Asia and the western Himalayas. *Asplenium ceterach* is more rare in northern Africa (Jahandiez and Maire, 1931; Maire, 1952; Quezel and Santa, 1962; Siddiqi, 1989), but extends into Eritrea and Somalia (Viane *et al.*, 1996), the Arabian Peninsula (Collenette, 1985), and Yemen (Christ, 1900; Wood, 1997). The autohexaploid *A. cyprium* Viane and Van den heede (Syn.: *A. ceterach* ssp. *cyprium* Viane) was described from Cyprus (Van den heede and Viane, 2002; Viane and Van den heede, 2002), and is also known from Greece and Sicily (Viane *et al.*, 1996; Van den heede *et al.*, 2002).

For the biosystematic revision of the *Ceterach* group (Van den heede, 2003), field trips were organized to study the Macaronesian representatives. Plants that we could not distinguish from the European *A. ceterach* were tentatively called *A. parvifolium*, and assumed to be octoploid. To our great surprise many of them turned out to be tetraploid.

In order to clear up the *A. ceterach–A. parvifolium* muddle on the Canarian Archipelago, we studied type material, and cytologically checked 145 samples from Gran Canaria, La Palma, and Tenerife. Because the type of *A. parvifolium* turned out to be *A. ceterach*, the octoploid taxon needed a new name and was described as *A. octoploideum* Viane and Van den heede (Van den heede and Viane, 2002).

Because electrophoretic analysis of isozymes has been successfully used in studies of reticulate complexes of Pteridophyta (Werth *et al.*, 1985a, 1985b; Werth, 1991; Haufler *et al.*, 1995) and has been applied at population and species levels (see Haufler, 1985b, 1997), we tried this method together with DNA sequencing, to determine whether true *A. ceterach* grows on the Canary Islands. A combination of morphological, cytological, and biogeographical data and isozyme markers can determine whether taxa are auto- or allo-polyploid (Crawford, 1985; Haufler, 1985b; Bryan and Soltis, 1987; Weeden and Wendel, 1989; Crawford, 1990; Pryer and Haufler, 1993). An overview of the literature about DNA sequencing in Pteridophyta is given in Van den heede *et al.* (2003).

Material and Methods

Between April 1995 and May 1999, field trips were organized to three Canary Islands from which *A. parvifolium* was known in the literature: Gran Canaria, La Palma and Tenerife. From 145 specimens, fronds with ripe spores were collected by C.V. and R.V, and ecological notes were made. Voucher information (Appendix 1, 2, and 3) is given only for specimens from which we were able to raise progeny and obtain cytological data.

The following localities are shown in Fig. 1:

1) Gran Canaria, S of Moya, "Los Tilos" Reserve, W exposed slopes of Barranco del Laurel, degraded laurel forest, in fissures of volcanic rocks; 28°05′03″N, 15°35′28″W, 600 m alt.
2) Gran Canaria, 4 km from junction Tejeda–San Mateo–Las Mesas, E exposed slopes of "El Nieblo" Nature Reserve, in fissures of volcanic rocks; 28°01′03″N, 15°36′07″W, 1550 m alt.
3) Gran Canaria, lava field near Cueva Corcho, along road GC110 from Artenara to Valleseco, 4 km NW of junction Artenara–Valleseco–Tejeda, in fissures of volcanic rocks; 1350 m alt.
4) Gran Canaria, 900 m S of Valsendero, W exposed cliff sides of narrow gully with laurel forest remnants; 28°02′48″N, 15°34′27″W, 900 m alt.
5) La Palma, c. 3 km E of Tijarafe, Pinar Lomo del Horno; 28°42′N, 17°55′W, 1140 m alt.
6) La Palma, S of Gallegos, Barranco Lomo de los Machines, Laurel forest W of tunnel El Envetadero, E exposed slope; 28°48′N, 17°50′W, 390 m alt.
7) La Palma, volcanic rocks above roadside to Fuencaliente, S of Monte de Luna; 28°31′N, 17°49′W, 710 m alt.
8) La Palma, footpath to Monte de Luna in Pinar S of Flores, in fissures of volcanic rocks; 28°31′N, 17°49′W, 810 m alt.

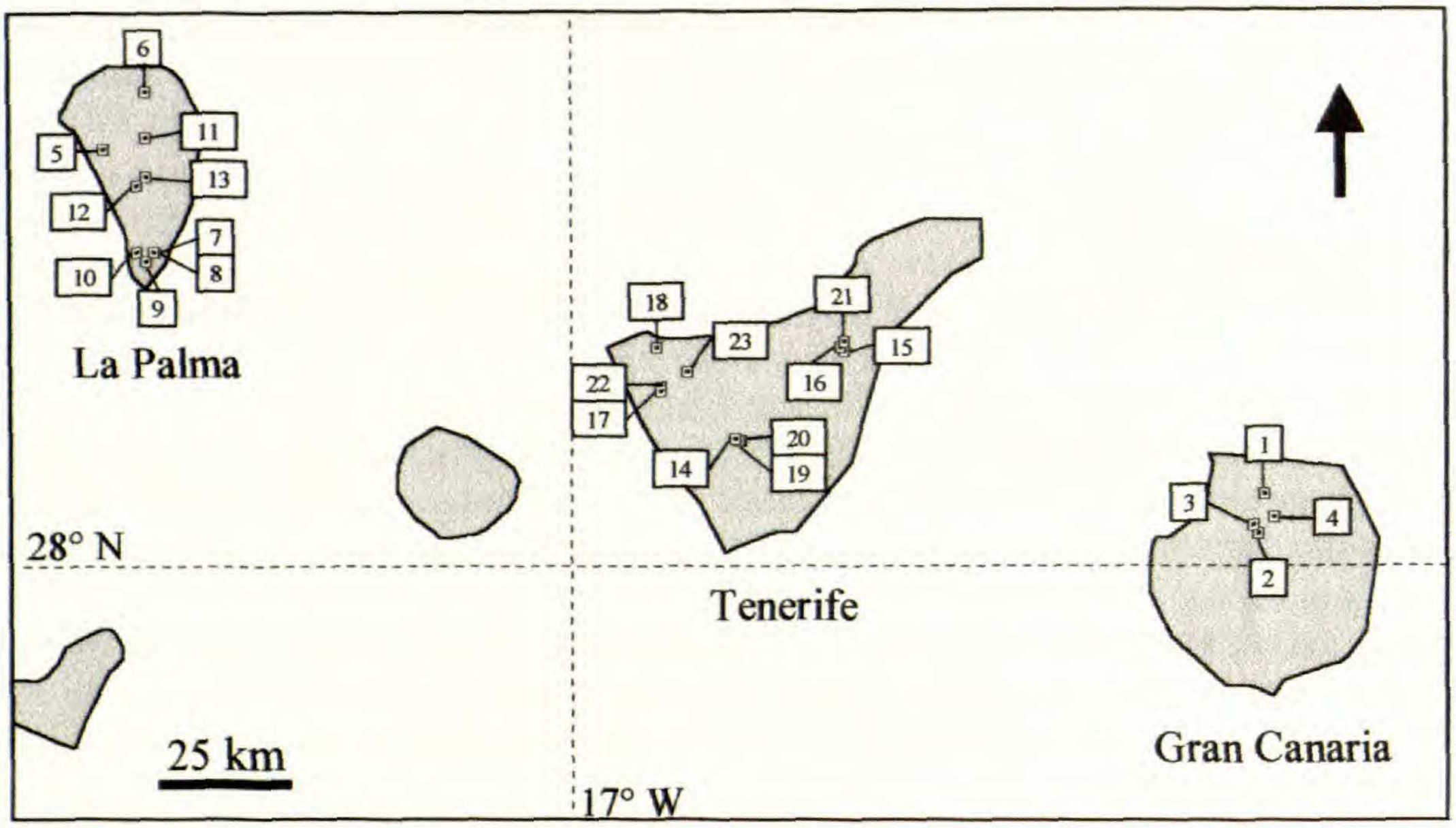

FIG. 1. Map of the western Canary Islands with localities of voucher specimens (see also Appendix 1, 2, and 3).

9) La Palma, along track to Pinar de la Virgen at junction with track to Caldera Los Arreboles, in fissures of volcanic rocks; 28°30′N, 17°50′W, 920 m alt.
10) La Palma, along track to Refugio de Tigalate from Zona Recreativa Fuente de los Roques, above "Malpais" W of Monte de Luna, in fissures of volcanic rocks; 28°31′N, 17°49′W, 1070 m alt.
11) La Palma, Caldera de Taburiente, track from La Cumbrecita to Hoyo de los Pinos, Pinar in Barranco de la Faya, in fissures of volcanic rocks; 28°43′N, 17°50′W, 1200 m alt.
12) La Palma, lava field 2.5 km E of El Paso church, in fissures of volcanic rocks; 28°38′N, 17°51′W, 800 m alt.
13) La Palma, lava field E of El Paso, NE of Montaña Las Moraditas, in fissures of volcanic rocks; 28°39′N, 17°50′W, 800 m alt.
14) Tenerife, along road from Vilaflor to Pico del Teide, ca. 8.1 km from junction Vilaflor–Santiago del Teide–La Orotava, under disc-like, SW exposed volcanic rocks; 28°10′55″N, 16°39′17″W, 1850 m alt.
15) Tenerife, Barranco de las Gambuesas above Arafo, N exposed slopes; 28°20′29″N, 16°26′02″W, 710 m alt.
16) Tenerife, Barranco del Espigon de Tea, NE exposed slopes; 28°20′44″N, 16°26′33″W, 825 m alt.
17) Tenerife, Montaña de la Hoya, ridge S of Las Manchas, above Ermita de la Santa Angel del Guardo, in fissures of volcanic rocks; 28°16′34″N, 16°48′05″W, 1120 m alt.
18) Tenerife, Teno, Barranco head between Tierra del Trigo and Ruigomez, along track above Tierra del Trigo, 1.8 km NW of Ruigomez, NW exposed basaltic slopes; 28°20′48″N, 16°48′28″W, 800 m alt.

19) Tenerife, Pinar above Vilaflor, Bandes de Chasna, c. 2.5 km NNW of Vilaflor, E exposed, in fissures of volcanic (phonolite) rocks; 28°10′48″N, 16°38′36″W, 1880 m alt.
20) Tenerife, Pinar above Vilaflor, W exposed slopes of small Barranco, in fissures of volcanic rocks; 28°10′48″N, 16°38′39″W, 1900 m alt.
21) Tenerife, Barranco de la Piedra Cumplida above (NW) Arafo; 28°21′17″N, 16°26′05″W, 900 m alt.
22) Tenerife, volcanic outcrop along footpath between Santiago del Teide and Arguayo, SE of El Retamar, between Montaña de la Hoya and La Hoya; 28°16″N, 16°48″W, 920 m alt.
23) Tenerife, lava field NW of Montaña de las Flores, "Vuelta Grande", along track from El Portillo del Rastrojo to Llanos del Hospital; 28°18″N, 16°45″W, 1410 m alt.
24) Tenerife, Chio Street, direction Cañadas, Restaurant "De Evora".

Vouchers listed in Appendix 1, 2 and 3, are deposited in the personal herbarium of Viane and Van den heede (including the T. Reichstein herbarium), with duplicates in GENT.

Between 1992 and 2001, R.V., C.V., and W. Bennert gathered additional material in Europe, Madeira, and Turkey (Appendix 4). Voucher information about 108 Cypriot samples is published in Van den heede *et al.* (2002). Our living European and Macaronesian *Asplenium* subg. *Ceterach* collection contained up to 550 specimens.

All material for this study has been cultivated in Ghent University Botanical Garden (Belgium). Spores were sown on agar-solidified medium containing a nutrient solution recommended by Dyer (1979). The cultures were stored in continuous light at room temperature. After formation of mature gametophytes, distilled water was added to achieve fertilization. If necessary, prothallia were transplanted onto fresh agar. Young sporophytes were planted individually in pots kept in a temperate greenhouse (minimum temperature 12°C). An air- and water-permeable soil mixture was required for these xerophytic rock ferns. Full-grown maturity was reached after approximately two years.

For chromosome counts, immature spore mother cells were fixed in the field, or in the greenhouse, using freshly prepared 3:1 absolute ethanol:glacial acetic acid, and stored at freezing-temperature until required. Acetocarmine squash preparations were made as described by Heitz (1925, 1950) and Manton (1950). Photographs were taken with an Olympus BH2 phase contrast microscope. Preparations were made permanent by dehydrating cover slip and slide in graded mixtures of acetic acid and absolute ethanol, followed by mounting in Euparal (T. Walker and H. Rasbach, pers. comm.). All permanent preparations are kept in the Pteridological Section of the Department of Biology at Ghent University. Sixteen cytologically checked plants (five tetraploids identified as *A. aureum*, seven tetraploids identified as "*A. parvifolium sensu* Benl," and four octoploids, (Appendix 1, 2, 3) were used as standards to compare the nuclear DNA content of the remaining specimens by a flow cytometer (Partec PA-1), using the manufacturer's protocol (Partec GmbH, Münster,

Nordrein-Westfalen, Germany). Both nuclei extraction solution and DAPI staining dilution were provided by Partec (Germany).

Methods for making permanent epidermis preparations and for measuring stomatal guard cells and spores, are described by Viane (1990, 1992). For exospore measurements untreated, fresh spores mounted in DePeX were used. Spore size is unaffected by DePeX, whereas in some other mounting media, e.g., glycerin-gelatin (Ormonde, 1990), spores expand by 5–15 %. The values of microcharacters are extracted from our regularly updated database, presently containing 110 different specimens of the European "*A. ceterach*" group, and 56 specimens of the Macaronesian "*A. aureum*" group (raw data available upon request).

Only vigorously growing plants were included in the allozyme study. Fresh leaves from 105 Canarian (Appendix 1, 2, 3) and 220 European and Turkish specimens (Appendix 4) were collected in the greenhouse, where the ferns were growing under the same conditions. Sporulating fronds of similar age were wrapped in wet tissue, stored in plastic bags, and kept refrigerated at 4°C for maximum 0.5–2 days (until extraction). Polyacrylamide gel electrophoresis (PAGE) was performed by C.V. at the laboratory of "General Botany and Nature Management" of the Free University Brussels (Belgium). Starch gel electrophoresis (SGE) was done by S.P. and E.P. in the "Departamento de Biologia Vegetal I" of the Universidad Complutense in Madrid (Spain). All specimens from the Canarian Archipelago were analysed by starch gel electrophoresis.

Equal amounts of tissue and extraction buffer were used to obtain uniform concentrations of extracts. Cooling (4°C) was applied during both homogenization and electrophoresis. Acquaah (1992) was consulted for the Enzyme Commission (E.C.) numbers.

PAGE procedures mentioned in Triest (1989) and Van den heede *et al.* (2002) were used, whereas SGE protocols followed Soltis *et al.* (1983) and Haufler (1985a). In a preliminary survey 19 enzyme systems (G-3PDH, G-6PD, GDH, IDH, MDH, ME, 6-PGD, SkDH, SOD, XDH, ACO, AAT, HK, PGM, β-EST, LAP, ALD, PGI, and TPI) were checked for polymorphism. Because the primary goal of this isozyme research was to test the hypothesis that tetraploid *A. ceterach* occurs on the Canary Islands in addition to *A. aureum* and related taxa, it was necessary to identify unique "marker" alleles characterizing each species or its progenitors. Finally, only five enzyme systems were suitable: aspartate aminotransferase (AAT = GOT, E.C. 2.6.1.1), shikimate dehydrogenase (SkDH, E.C. 1.1.1.25), malic enzyme (ME, E.C. 1.1.1.40), phosphoglucose isomerase (PGI, E.C. 5.1.3.9), and triosephosphate isomerase (TPI, E.C. 5.3.1.1). All pictures and dried gels are kept in the Pteridological Section of the Department of Biology at Ghent University.

Band homologies were determined by running samples side-by-side on the same gel (see Haufler *et al.*, 1995). Allelic variants within loci were distinguished from the products of different loci by assuming that *Asplenium* enzymes conformed to established models of organellar compartmentalization (Gottlieb, 1982; Gastony and Darrow, 1983; Soltis, 1986; Weeden and Wendel, 1989). Presumed loci were numbered sequentially, with the most anodally

(i.e., the fastest band) migrating one designated "1." Similarly, different alleles of the same gene locus (i.e., allozymes, Crawford, 1990) were denoted alphabetically with the most anodal being "*a*."

Sequencing work was done by C.V. at the Jodrell Laboratory in Kew (United Kingdom). Nineteen cytologically and isozymically interesting, vigorously growing plants, including three *A. aureum* specimens (*CV164, CV670, CV712*) from Gran Canaria, Tenerife, and La Palma, one putative *A. ceterach* from Tenerife (*CV187*), and one octoploid from La Palma (*CV709*), were selected to generate DNA sequences from the plastid *trnL-trnF* intergenic spacer. European material for comparison included two *A. javorkeanum* specimens from Italy and Slovenia (*CV14* and *CV85b*), two *A. ceterach* samples from Italy and Cyprus (*CV494* and *CV225*), and a hexaploid *A. cyprium* plant (*CV249*) from Cyprus (see Appendix 4). Sequences of the closely related *A. lolegnamense* (Gibby and Lovis) Viane from Madeira (*CV985 and CV993*), of the less related *A. dalhousiae* Hook. from Ethiopia and Pakistan (*CV318 and TR7634*), and of the more distantly related *A. nidus* L. (*AF425118*), and *A. scolopendrium* L. and *A. unilaterale* Lam. (R. Cranfill, University of California, Berkeley, California, USA, unpublished data) were included as outgroups. The *trnL-F* sequence of a species of *Dennstaedtia* (R. Cranfill, unpublished data) was used to represent a group basal to the Aspleniaceae (e.g., Bower, 1928; Christensen, 1938; Copeland, 1947; Pichi Sermolli, 1977; Kramer and Green, 1990; Hasebe *et al.*, 1995; Pryer *et al.*, 1995). Methods are explained in Van den heede *et al.* (2003).

Results and Preliminary Discussion

To avoid prolixity, we have combined both the results and the interpretation of the isozyme phenotypes.

Chromosomes were counted for 16 specimens collected on Gran Canaria, La Palma, and Tenerife. In addition to five tetraploid *A. aureum* ($n = 72^{II}$) plants, eleven small specimens that we could not distinguish from European *A. ceterach*, were examined. Seven of them turned out to be tetraploid ($n = 72^{II}$) and four were octoploid, having a meiotic chromosome number of $n = 144^{II}$ (Fig. 2). Meiosis in all cells examined was regular, showing only bivalents, and giving no indication about the polyploid status of the species. This agrees with Lovis' (1977) statement that most autopolyploid ferns possess diploidized meiosis (only bivalent formation), and "that the absence of multivalents is no valid evidence of allopolyploidy."

The counted samples were used as standards to determine the ploidy level of the remaining 146 specimens using a flow cytometer. Results are given in Appendix 1, 2 and 3; localities of cytologically checked material are indicated on the map of the Canarian Archipelago (Fig. 1).

In May 1995 (*RV6135*) and 1997 (*CV165–170*; *CV183–187*), we discovered tetraploid *A. ceterach* specimens on both Gran Canaria and Tenerife (see Appendix 1 and 3).

The three species (*A. aureum*, the "small tetraploid", and the octoploid) cannot always be distinguished macromorphologically, but can be identified

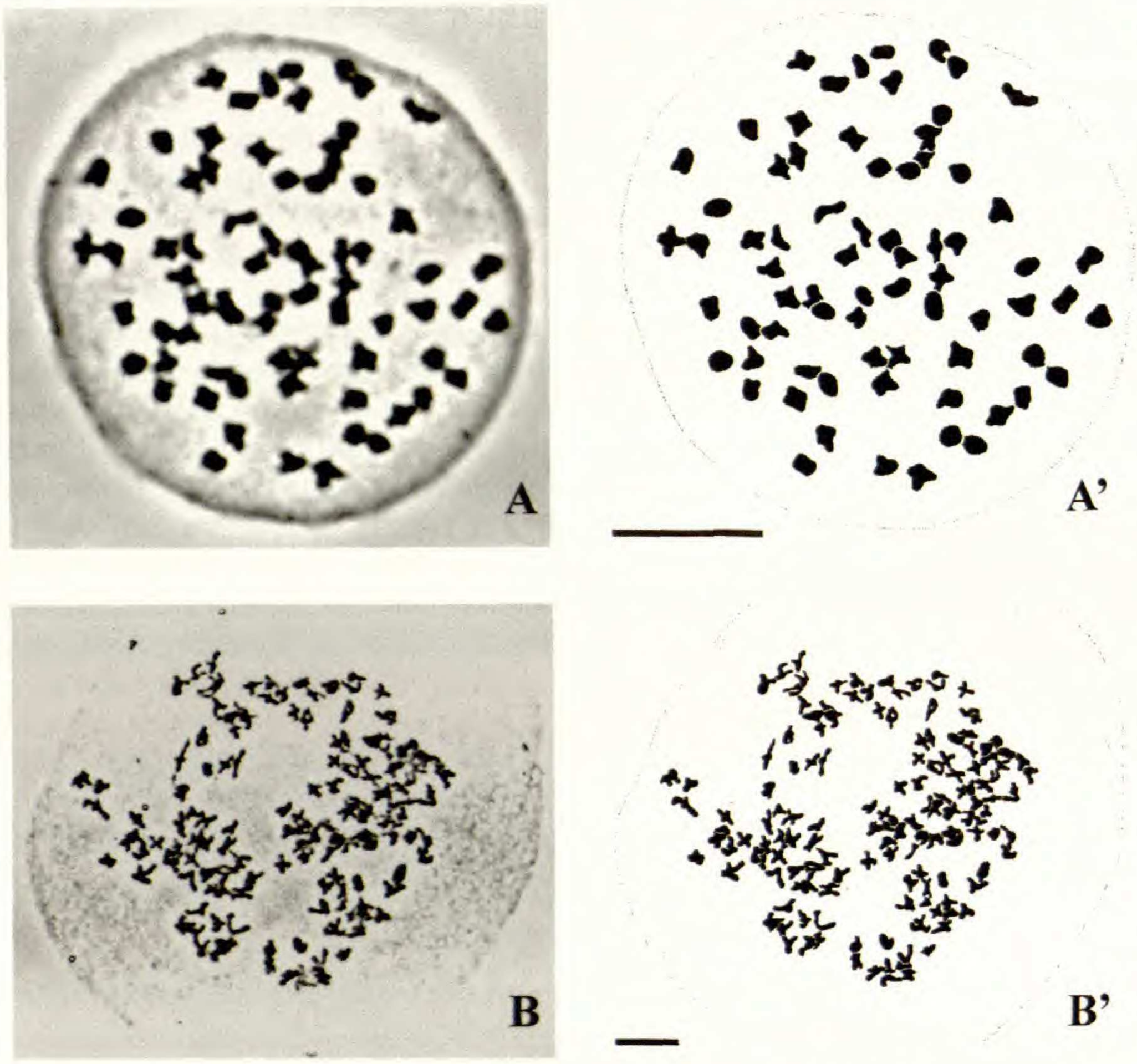

FIG. 2. Cytology showing spore mother cells in first meiotic division. A, B = photographs; A', B' = explanatory diagrams with bivalents in black.
A, A': *A. ceterach* (CV 170b), metaphase I showing n = 72^{II}. B, B': *A. octoploideum* (CV 188, holotype), cell showing n = 144^{II}. Scale bar = 10 μm. (preparations, photos and diagrams: C.V.).

by measuring exospore length (Table 2). Stomatal guard cell length can only be used to distinguish the "small tetraploid" (45 ± 3.8 μm) from the octoploid (52 ± 4.8 μm). We found no differences in perispore morphology, stomatal type, or epidermal cell pattern. Perispores have costato-cristate folds with few perforations, stomates are mesopolocytic, and epidermal cells mostly sinuous. Polyploidy factors (Viane, 1986, 1990) in *A. ceterach* are $P_{cet,\ exo} = 1.25$ (for the exospore) and $P_{cet,\ sto} = 1.16$ (for the stomates), and $P_{aur,\ exo} = 1.18$ and $P_{aur,\ sto} = 1.07$ in *A. aureum*. Using these P-values, the theoretical spore and stomate sizes calculated for the octoploid are less than 1 s.d. different from their actual means (Table 2), thus supporting the proposed ancestry (Viane, 1990).

We stress the presence of a small indusium in all taxa; it can best be observed in epidermis preparations (Viane, 1990). Our observations show that the (toothed) margin and the "cuticular lines" of the scales, are unreliable characters to

TABLE 2. Microcharacters differentiating taxa within the *A. aureum–ceterach* group on the Canary Islands. All measurements are based on cytologically checked material. Additional information about material and the number of measurements is available from the authors.

Taxon	Ploidy	Mean exospore length ± s.d.	Mean guard cell length ± s.d.
A. aurem	4x	32 ± 1.9 μm	46 ± 4.4 μm
A. ceterach	4x	39 ± 2.6 μm	45 ± 3.8 μm
A. octoploideum	8x	44 ± 3.1 μm	52 ± 4.8 μm

discriminate *A. aureum* and relatives from *A. ceterach*. All taxa have scales with more or less dentate margins, and periclinal cell walls with or without "cuticular lines". These "cuticular stripes" are folds in the periclinal cell wall (Fig. 3), and the bigger the cell the more folds seem to be present. However, *A. aureum* scales usually show numerous folds, whereas *A. ceterach* (from its entire range of distribution) paleae possess only few. In the octoploid the number of folds is usually intermediate between that in *A. aureum* and *A. ceterach*.

Isozyme analysis can be used to determine whether taxa are auto- or allopolyploid. The electrophoretic phenotype of an autopolyploid should show a subset of the isozymes present in its progenitor, assuming no mutation subsequent to the origin of the polyploid (Weeden and Wendel, 1989; Crawford, 1990; Pryer and Haufler, 1993). An allopolyploid should display fixed heterozygous (i.e., nonsegregating) banding patterns for many loci, resulting from the combination of different parental genomes (Gottlieb, 1982; Werth, *et al.* 1985b; Pryer and Haufler, 1993; Soltis and Soltis, 2000). Fixed heterozygous banding patterns differ from normal heterozygous zymograms, because the bands do not segregate among progeny and remain fixed in all specimens.

Nineteen enzyme systems were tested in a preliminary survey. The low resolution of ALD, and the smeared patterns of XDH made both unusable. IDH,

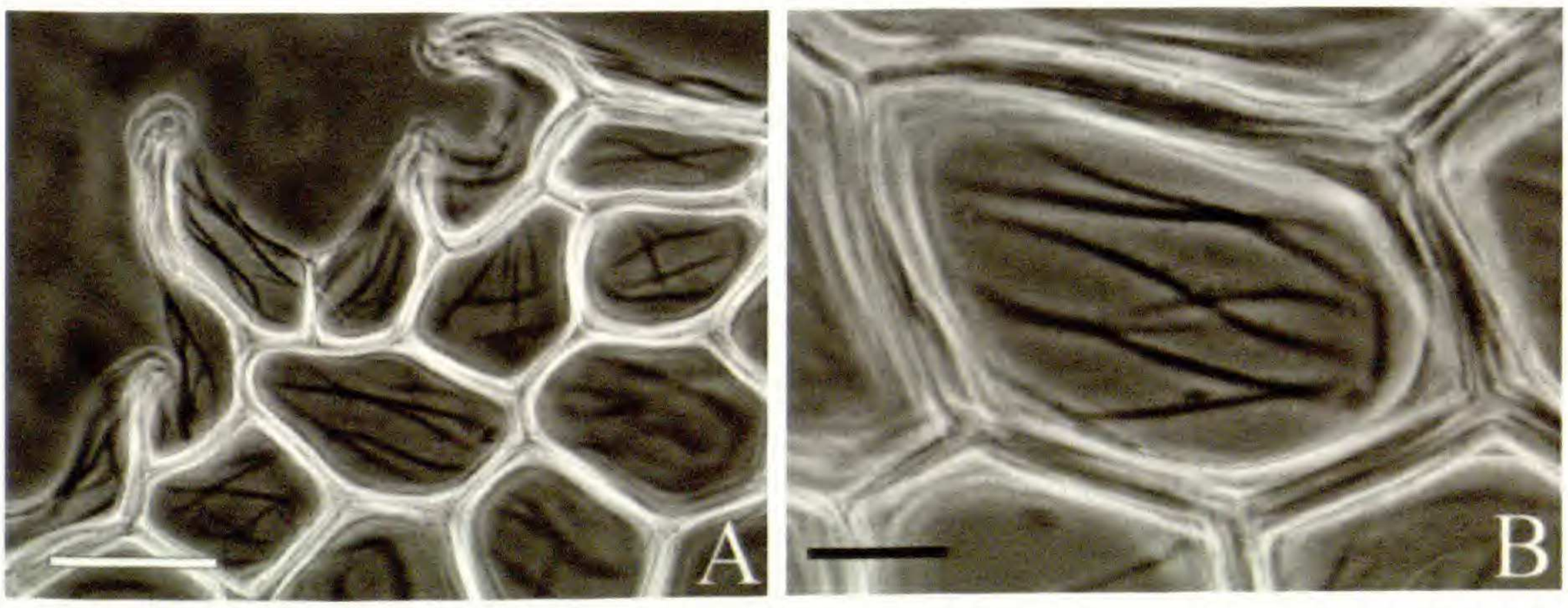

FIG. 3. Folds ("cuticular lines") in periclinal cell walls of *A. aureum* paleae. Phase contrast micrographs of laminal scales (CV 157). A: scale margin with several cells. B: single cell with folds. Bar: A = 50 μm, B = 10 μm.

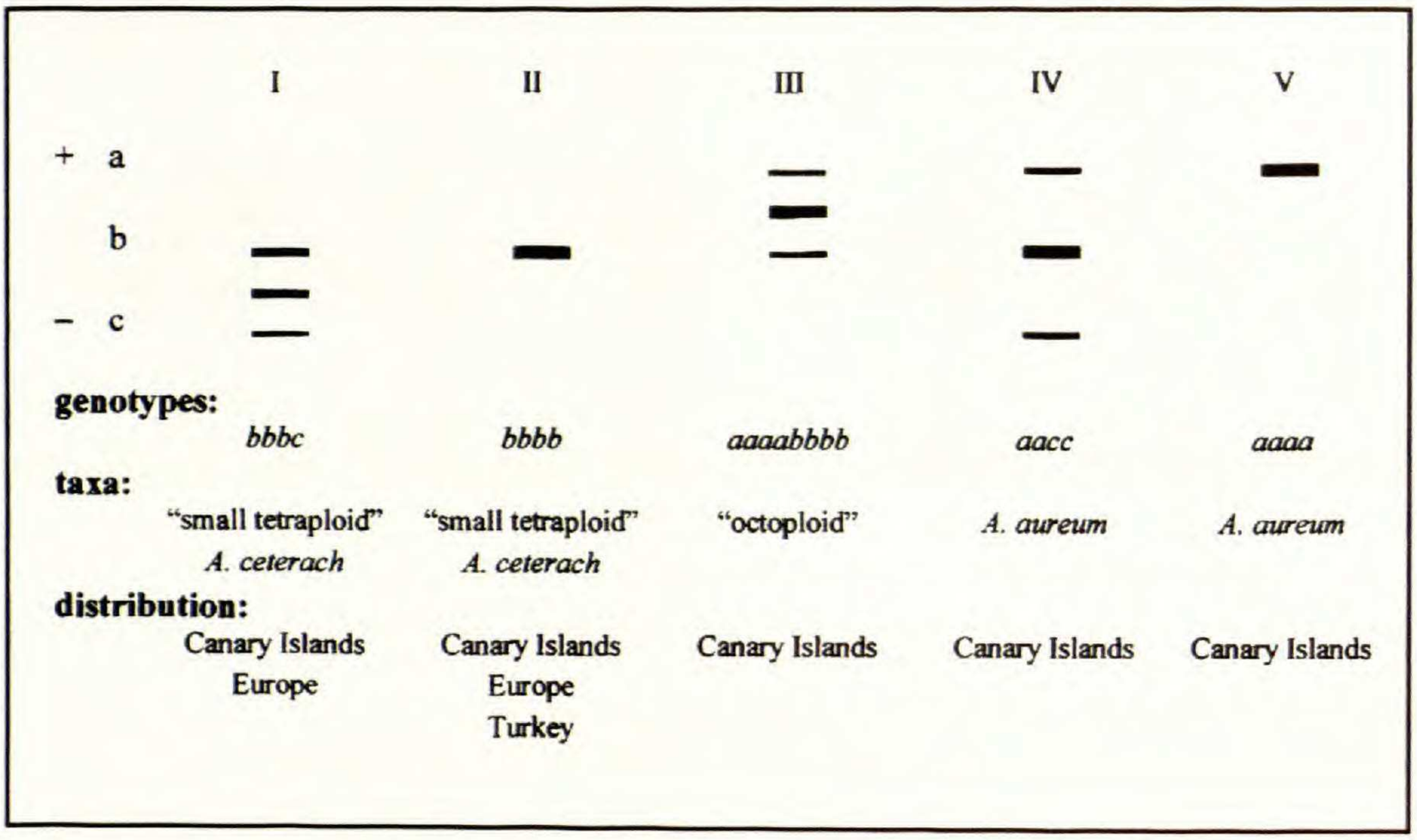

FIG. 4. Diagrams of electrophoretic AAT phenotypes, showing 3 alleles and the corresponding genotypes, as observed in the European–Canarian *Asplenium ceterach–aureum* group. Zymotype II was also found in all samples of *A. javorkeanum* (*bb*) and *A. cyprium* (*bbbbbb*) examined.

MDH, ACO, and SOD yielded unclear patterns with limited variation. G-6PD and G-3PDH gave inconsistent zymograms. Consequently, these enzyme systems were not retained.

To test our hypothesis that in addition to *A. aureum* and the octoploid, true *A. ceterach* occurs on the Canary Islands, the following four enzyme systems were suitable: AAT, SkDH, ME, and PGI. These enzyme systems yielded reproducible, well resolved banding patterns discriminating specimens representing *A. aureum*, *A. ceterach* and the allo-octoploid hybrid. These enzymes, encoded by four putative loci, were also used to get an idea of the variation of the species.

AAT or GOT: this dimeric enzyme was studied using both PAGE and SGE (system 8 of Haufler, 1985a). We observed only one activity zone, which agrees with Gastony and Darrow (1983), who proved that this single enzyme activity is chloroplastic.

Most of the specimens studied are homozygous, showing a single well-resolved AAT band (Fig. 4, zymotype II). This applies to all 50 *A. javorkeanum* specimens (Italy and Slovenia), most (102) *A. ceterach* plants (Belgium, Croatia, Cyprus, France, Italy, Slovenia, Spain, Turkey, and the United Kingdom), 23 "small tetraploids" from the Canary Islands, and all (44) *A. cyprium* samples tested (Van den heede *et al.* 2002). Ten *A. ceterach* individuals from Croatia, France, Italy, and Slovenia, and two "small tetraploids" from the Canary Islands, showed the rarer three-banded zymotype I with skewed staining intensities, interpretable as heterozygous for a dimer.

Corresponding genotypes are *bb, bbbb,* or *bbbbbb* for zymotype II of the homozygous (diploid to hexaploid) plants, versus *bbbc* for zymotype I of the heterozygous tetraploid specimens.

Identical banding patterns in the Canarian "small tetraploid" and in European *A. ceterach* plants indicate that true *A. ceterach* is growing on the Canary Islands. Because neither European nor Canarian samples reveal unique electrophoretic phenotypes, the Canarian populations do not seem to be genetically isolated. Though more sampling of *A. javorkeanum* is needed, these preliminary results (Fig. 4, zymotype II) seem to confirm the autotetraploidy of *A. ceterach*.

The *a* allele (Fig. 4, zymotype V) can be used as a "marker" allele characterizing *A. aureum*. Some plants, showing a single band corresponding to genotype *aaaa*, are homozygous, whereas others, with a balanced three-banded zymogram corresponding to genotype *aacc*, are heterozygous. To explain zymotypes I and IV, an extra genotype *cc* is postulated and expected in *A. javorkeanum*, which was studied only on the basis of Italian and Slovenian material.

All 54 octoploid specimens (from Gran Canaria, La Palma, and Tenerife) show a monomorphic, presumably fixed heterozygous banding pattern of genotype *aaaabbbb* (Fig. 4, zymotype III), which we postulate to be derived from a combination of zymotypes II and V (Fig. 4). This would agree with the suggestions of Reichstein (1984) and Viane and Reichstein (1992), that the Canarian octoploid is an allo-octoploid, which originated either by chromosome doubling in an unknown tetraploid hybrid between *A. aureum* (with zymotype V) and *A. ceterach* (with zymotype II), or via unreduced gametes of each species (Fig. 9). The fact that, in all the octoploids (from 15 different localities) only one AAT zymotype was detected can be explained by the preponderance of the "small tetraploid" with zymotype II. It may also reflect incomplete sampling of the variation present in the octoploid.

SkDH: resolution for this monomeric enzyme was superior on SGE (system 2 of Weeden and Wendel, 1989). In our study, the enzyme was represented by a single locus, which agrees with Gastony and Darrow (1983). As expected for a monomeric enzyme, homozygotes had a typical one-banded pattern whereas heterozygotes showed two or more bands.

We detected four alleles in the European–Macaronesian *Asplenium ceterach–aureum* group. Two of these, *a* and *b*, were observed in *A. aureum*, whereas *c* and *d* characterized the "*A. ceterach*" group. Although SkDH was polymorphic in *A. ceterach* (Fig. 5), only zymotype V (*cccd*) was found on the Canary Islands. This two-banded pattern with unequal staining intensities forms also part of zymotype VI found for all 54 octoploid specimens. Thus the octoploid is monomorphic and presumably heterozygous for this locus, showing a four-banded zymogram corresponding to genotype *aabbcccd*. The *a* and *b* alleles are unique "marker" alleles for *A. aureum*, one of the progenitors of the octoploid. This monomorphic pattern showing presumed fixed heterozygosity seems to confirm the putative allo-octoploid origin of this species (Reichstein, 1984; Viane and Reichstein, 1992). The *cccd* SkDH

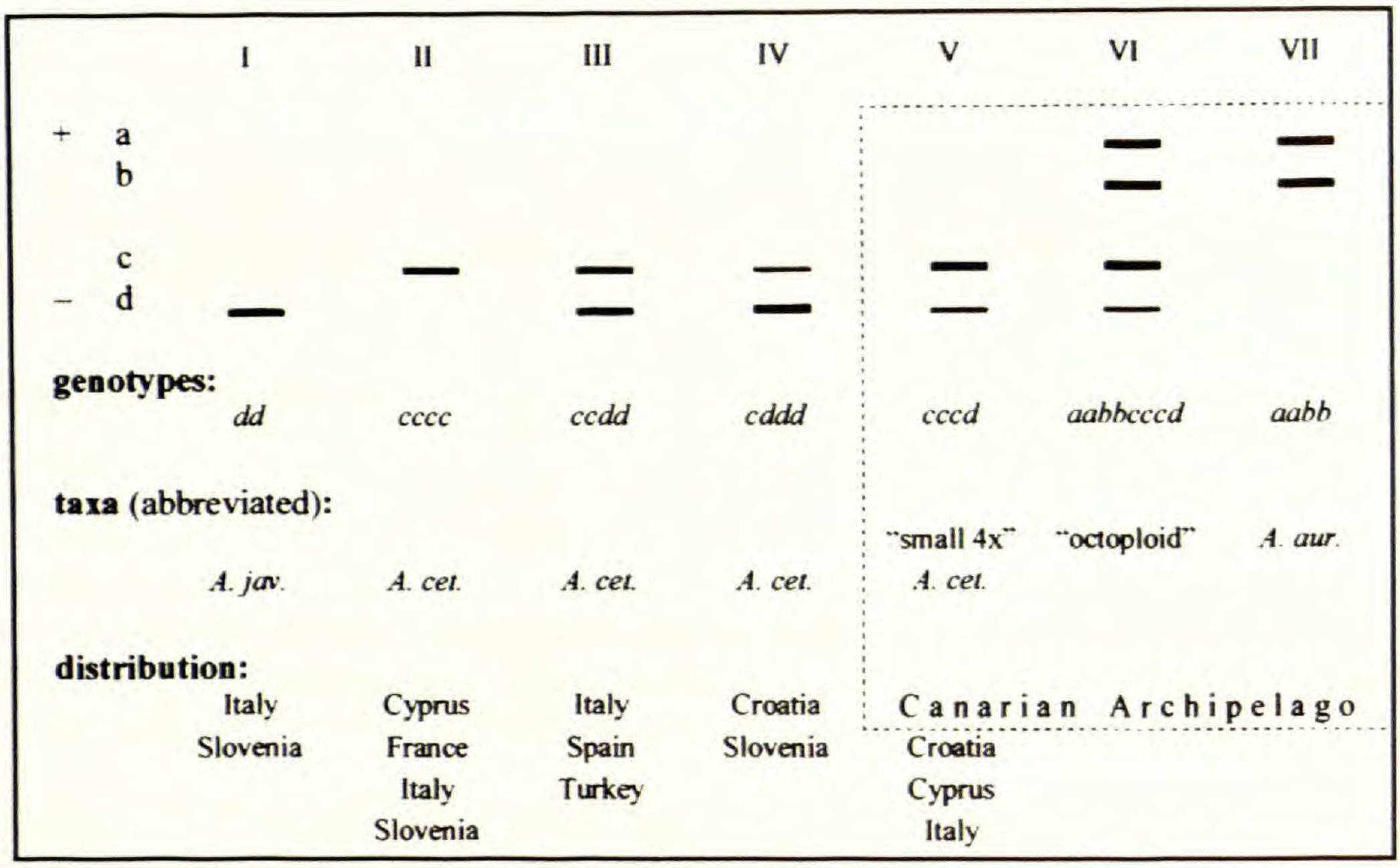

FIG. 5. Diagrams of electrophoretic SkDH phenotypes, with corresponding genotypes, as observed in the European–Canarian *Asplenium ceterach–aureum* group. Zymotype II was also found in some *A. javorkeanum* (genotype: *cc*); zymotype V was present in all *A. cyprium* (genotype: *ccccdd*) samples checked. The Canarian small tetraploid is abbreviated as: "small 4x."

genotype (zymotype V) of the "small tetraploids" is not limited to the Canaries, but was also found in *A. ceterach* from Croatia, Cyprus, and Italy. These results again both prove the occurrence of *A. ceterach* on the Canary Islands, and the fact that the populations in this Archipelago are not genetically isolated. Three additional zymotypes were detected in continental *A. ceterach*: a single-banded (*cccc*), a balanced two-banded (*ccdd*), and an unbalanced two-banded pattern (*cddd*). The presence of the unbalanced patterns (*cccd, cddd*) in tetraploid *A. ceterach* at *Skdh* can be explained by tetrasomic inheritance (see discussion). Diploid *A. javorkeanum* from Italy and Slovenia showed a single-banded pattern of either genotype *cc* (zymotype II) or *dd* (zymotype I).

ME: this tetrameric enzyme was studied only by PAGE. The single enzyme activity visible was shown to be cytosolic by Gottlieb (1982), Gastony and Darrow (1983), and Soltis (1986).

ME was monomorphic in each of the three species, and thus can be used to distinguish them from each other (Fig. 6). All *A. ceterach* specimens (Europe) and "small tetraploids" (Canary Islands) were heterozygous showing an identical five-banded zymogram, typical for a tetrameric enzyme controlled by one locus with two alleles, *a* and *d*. Heterozygous *A. aureum* was characterized by a five-banded pattern controlled by the same locus, but with two different alleles, *b* and *c*, and corresponding to genotype *bbcc*. All octoploid plants

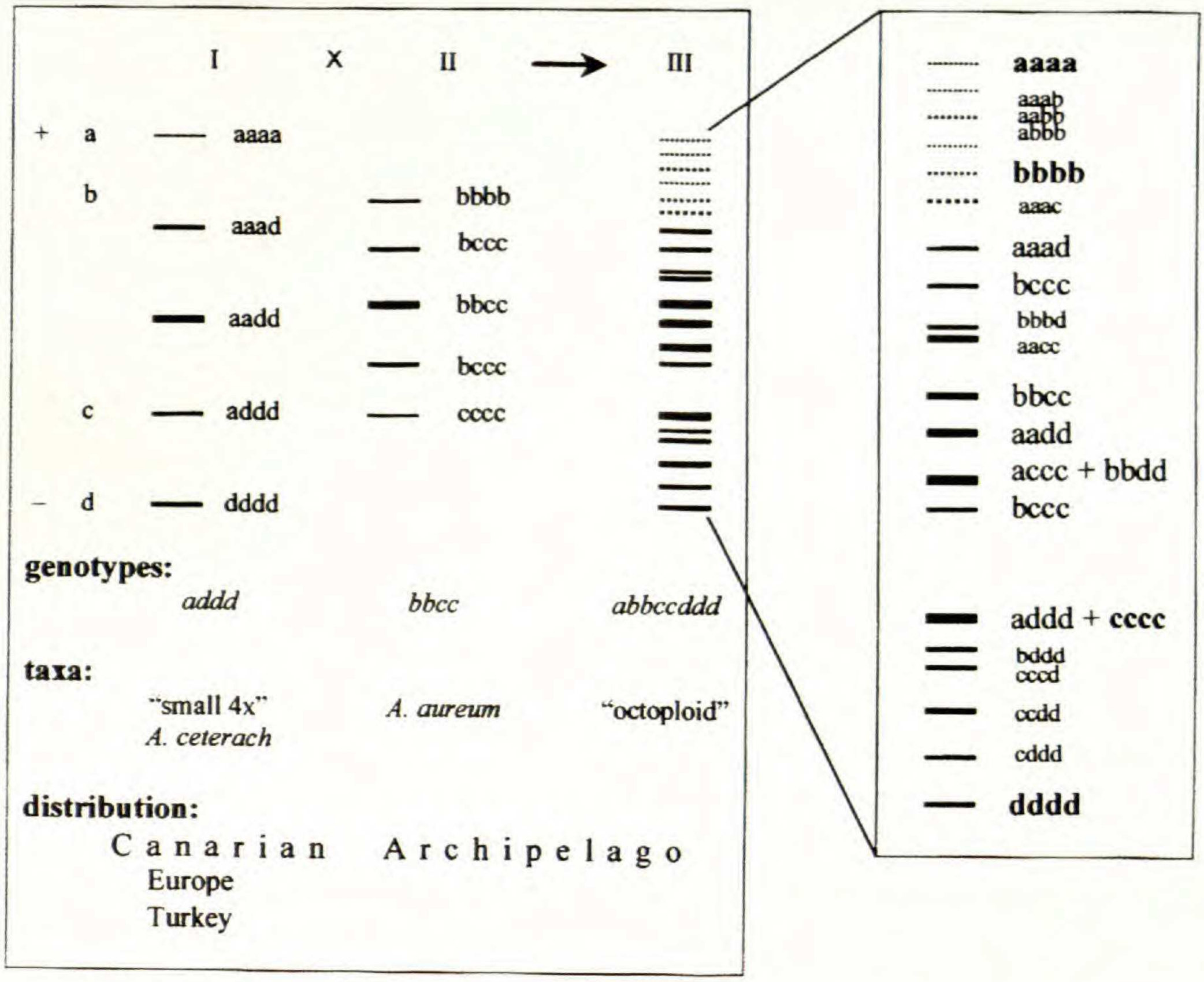

FIG. 6. Diagrams explaining the ME zymotypes showing 4 alleles, with corresponding genotypes, as observed in the European–Canarian *Asplenium ceterach–aureum* group. For each band four letters represent the association of subunits (coded by alleles) joined to form this tetrameric enzyme. The homotetramers in the "hybrid" pattern are in boldface. Zymotype III confirms the allopolyploid status of the octoploid. Dotted lines indicate very faint bands. The Canarian small tetraploid is abbreviated as "small 4x."

showed a complex zymogram, and conform to the expected hybrid phenotype resulting from the cross between *A. ceterach* and *A. aureum*. The "hybrid" had the four parental alleles, and since ME is a tetramer, each of the six pairs of alleles ($a \times b$, $a \times c$, $a \times d$, $b \times c$, $b \times d$, $c \times d$) formed three heterotetramers of intermediate mobility. Theoretically this results in a 22-banded pattern (4 homotetramers plus $6 \times 3 = 18$ heterotetramers, makes 22 bands), but because twice two bands have the same mobility, a maximum of 20 bands was visible (Fig. 6). The monomorphic and presumably fixed banding pattern of the "hybrid" zymogram is in agreement with the putative allopolyploid origin of the octoploid (Reichstein, 1984; Viane and Reichstein, 1992).

PGI: this dimeric enzyme was studied by both PAGE and SGE. Because the resolution was much better with SGE, all results shown were obtained using starch gel electrophoresis (system 6 of Soltis *et al.*, 1983).

Two loci were present: *Pgi-1*, most probably chloroplastic, and *Pgi-2*, cyto-

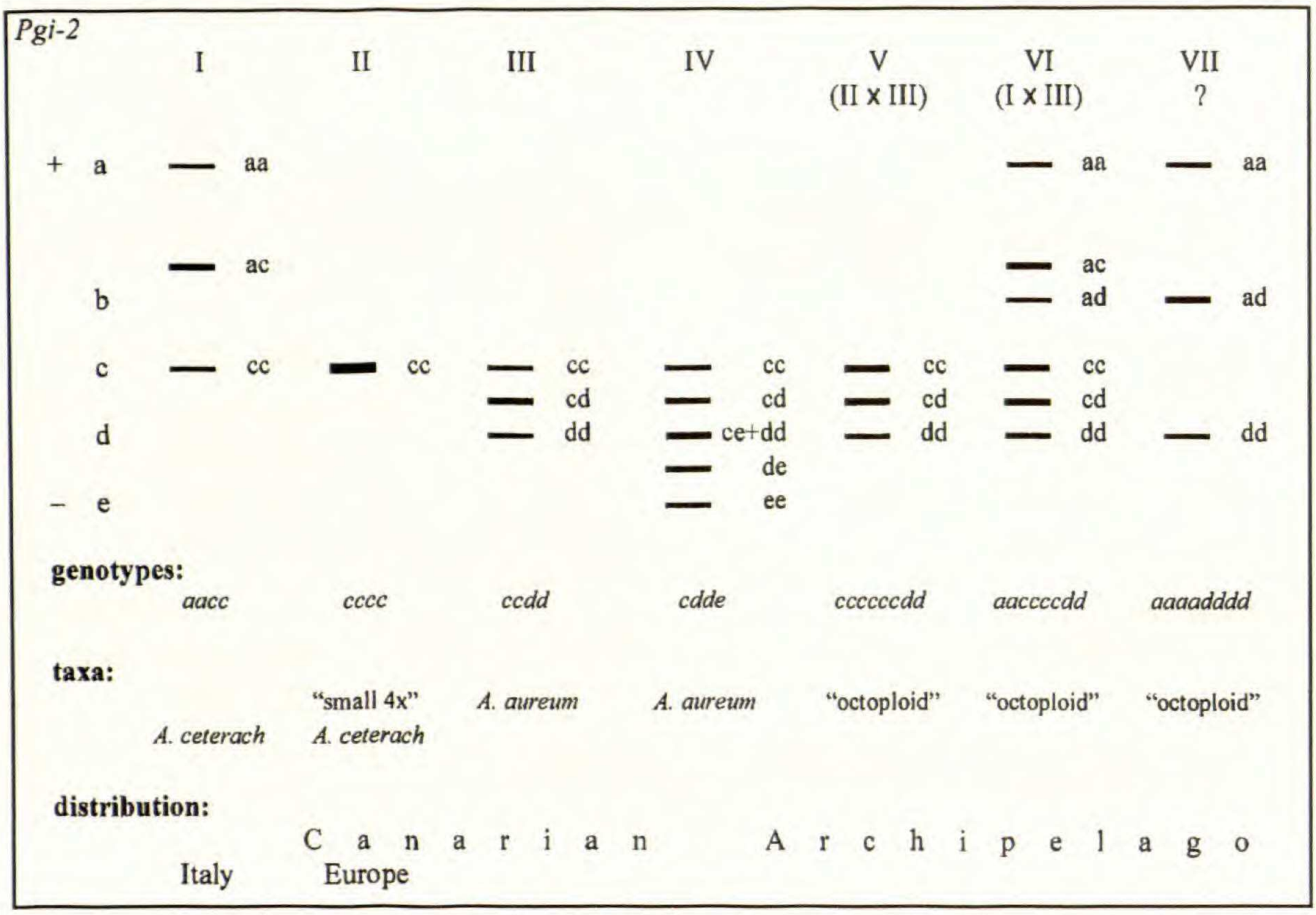

FIG. 7. Diagrams of electrophoretic *Pgi-2* phenotypes, showing 5 alleles, with corresponding genotypes and their distribution, as observed in the European–Canarian *Asplenium ceterach–aureum* group. Zymotype II was also found in most *A. javorkeanum* (*cc*) samples. Each band is indicated by two letters representing the association of subunits, joined to form this dimeric enzyme. The Canarian small tetraploid is abbreviated as "small 4x."

solic (Gastony and Darrow, 1983; Soltis, 1986). Consistent with observations on other ferns (Gastony and Gottlieb, 1985; Werth, 1991; Haufler *et al.*, 1995; Hauk and Haufler, 1999), resolution of the more anodal locus *Pgi-1* was inferior to that of *Pgi-2*. Because *Pgi-1* appears invariant across all taxa, it will not be discussed.

Among the European and Macaronesian samples studied, five allozymes were observed at *Pgi-2* (Fig. 7). Although the continental *A. ceterach*, with its six different zymotypes, was highly polymorphic for this locus (Van den heede *et al.*, 2002), only a single banding pattern was detected for the 22 "small tetraploids" from the Canary Islands, corresponding to genotype *cccc*. This widely distributed zymotype was also found in *A. ceterach* specimens from Belgium, Croatia, France, Italy, Slovenia, Spain, and the United Kingdom. We obtained two electrophoretic phenotypes for the 28 *A. aureum* plants, with corresponding genotypes *ccdd* (25 specimens) and *cdde* (3 specimens). The octoploid was the most variable taxon in the Canarian Archipelago, showing three different zymotypes translated into genotypes (Fig. 7) *ccccccdd* (zymotype V), *aaccccdd* (zymotype VI), and *aaaadddd* (zymotype VII). Zymotype V (found only on La Palma) most probably resulted from hybridization

TABLE 3. Genbank accession numbers for *trnL-trnF* nucleotide sequences of newly sequenced *Asplenium* specimens. CV and TR are abbreviations for Caroline Van den heede and Tadeus Reichstein respectively. Localities are given in Appendix 4.

Species	Voucher number	Data of collection	GenBank accession number
A. aureum	*CV164*	25 May 1997	AY160993
	CV670	12 Jan. 1999	AY160994
	CV712	3 Apr. 1999	AY160995
A. ceterach	*CV187*	27 May 1997	AY162333
	CV225	11 June 1997	AY162334
	CV494	17 Aug. 1998	AY162335
A. cyprium	*CV249*	12 June 1997	AY162337
A. dalhousiae	*CV318*	13 Jan. 1998	AY161000
	TR7634	27 Aug. 1990	AY161001
A. javorkeanum	*CV14*	24 July 1996	AY162330
	CV85	30 Aug. 1996	AY162331
A. lolegnamense	*CV985*	29 May 2000	AY160998
	CV993	1 June 2000	AY160999
A. octoploideum	*CV709*	2 Apr. 1999	AY161003

between a "small tetraploid" with genotype *cccc* and an *A. aureum* with genotype *ccdd*, which are both abundantly present on the Canaries, followed by chromosome doubling, or via unreduced gametes of each species. Zymotype I, though presently known only from Italy, can be used to explain zymotype VI, which was found only on La Palma. Octoploids with this genotype (*aacccccdd*), expressed three homodimeric bands (Fig. 7, aa, cc, dd) plus three heterodimeric bands (ac, ad, cd). More sampling is desirable and might detect other genotypes such as *aacc* in the "small tetraploid," as well as *dddd* needed to explain zymotype VII from Gran Canaria and Tenerife. *Pgi-2* suggests that the formation of the allo-octoploid happened at least three times.

Because plastid DNA is uniparentally inherited, it discloses only the maternal lineage (Stein and Barrington, 1990; Gastony and Yatskievych, 1992). GenBank accession numbers for *trnL-trnF* nucleotide sequences of newly sequenced specimens are listed in Table 3. Analysis of the plastid *trnL-trnF* intergenic spacer sequences resulted in the clustering of the "small tetraploid" from Tenerife (*CV187*), *A. ceterach* from Italy and Cyprus, and *A. cyprium*, with their diploid ancestor *A. javorkeanum* (Fig. 8). We found no chloroplast variation (with the exception of *CV494*) between specimens sampled from the Mediterranean (Cyprus, Italy, Slovenia) and Tenerife. *Asplenium javorkeanum*, *A. ceterach*, and *A. cyprium* form a cluster of their own, different from the "*A. aureum* clade," which includes all the *A. aureum* specimens, *A. lolegnamense*, and the octoploid (*CV709*) from the Canaries. Identical groups are obtained by analysing *rbcL* gene sequences. The position of *A. lolegnamense* and the octoploid, in the plastid trees, suggests that *A. aureum* acted as the maternal parent in the formation of the specimens used. These molecular data independently prove that in addition to an octoploid species, true *A. ceterach*

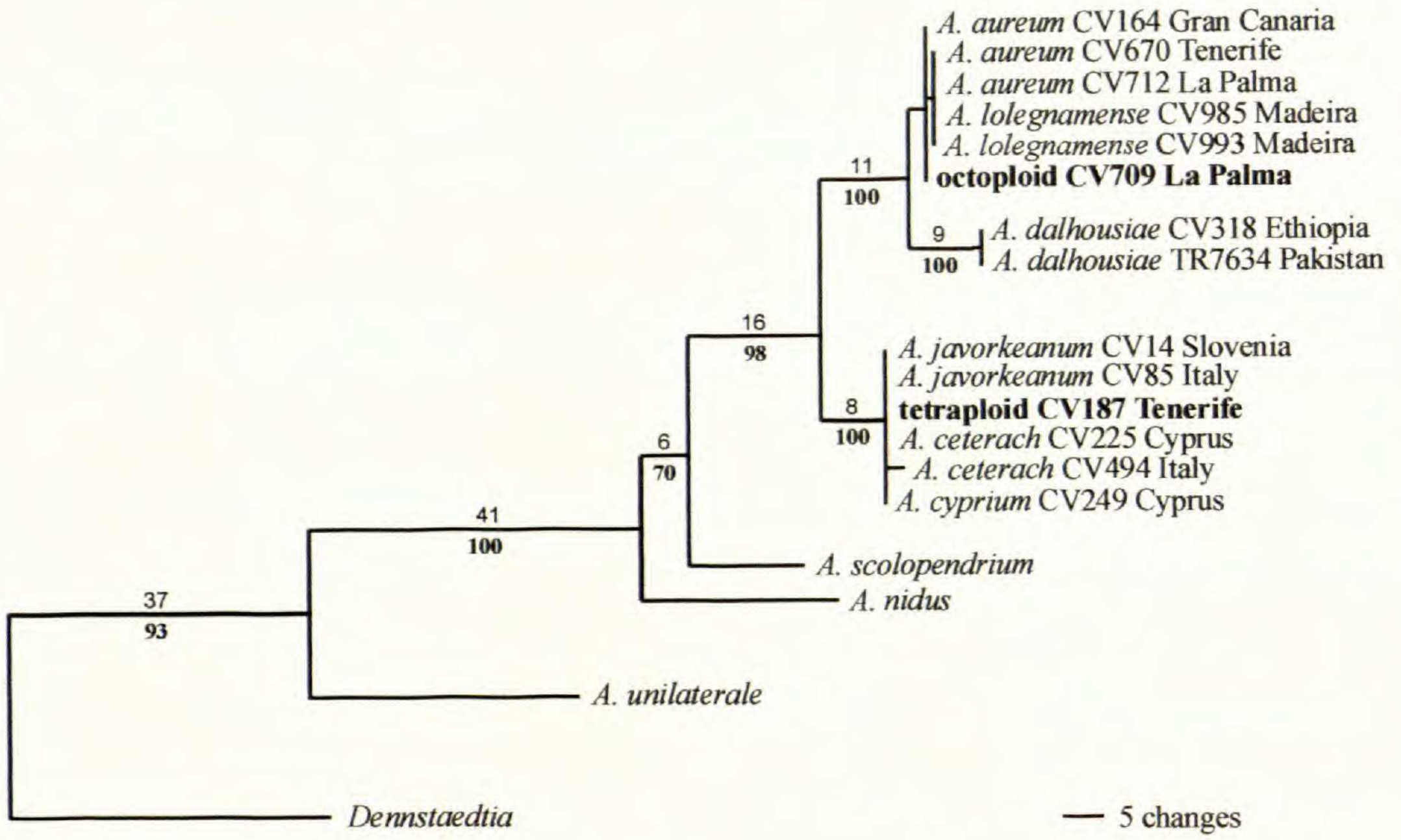

FIG. 8. Tree randomly selected from the 73 shortest trees of European–Canarian *Asplenium ceterach–aureum* taxa and *A. dalhousiae*, resulting from parsimony analysis of our 14 *trnL-trnF* intergenic spacer sequences (Table 3) and 4 *trnL-trnF* sequences of other species available in GenBank; length = 227 steps, CI = 0.92, and RI = 0.91. Based on *rbcL* evidence (Hasebe et al., 1995; Pryer et al., 1995), the sequence of the more distantly related *Dennstaedtia* was specified as outgroup. Fitch branch lengths (ACCTRAN optimized) are shown above and bootstrap percentages (1000 replicates) below the branches. Other sequencing results are described extensively in Van den heede et al. (2003).

(= the "small tetraploid") is growing on the Canary Islands. Other sequencing results are described extensively in Van den heede *et al.* (2003).

Because Gran Canaria, La Palma, and Tenerife are of volcanic origin, these epilithic ferns mainly grow on rocks of basaltic types, like phonolites, rhyolites, trachytes and olivine basalts (Page, 1979). *Asplenium aureum* prefers moister, shady habitats at lower altitudes, whereas the "small tetraploid" and octoploid plants share more exposed, drier habitats. However, on both Gran Canaria and Tenerife, only single localities were found where "small tetraploids" and octoploids grew together (loc. 2 and 19; see Appendix 1 and 3). Though forty-five specimens from nine different localities on La Palma were cytologically checked (see Appendix 2 and Van den heede and Viane, unpublished data), we could not detect any "small tetraploid" specimen. We intensively looked for it in the field, especially in the higher regions of La Palma. Whereas we discovered "small tetraploids" between 1500 and 1900 m altitude on Gran Canaria and Tenerife, the highest altitude we found ferns of the *Ceterach* group on La Palma was near 1200 m.

We found *A. aureum* between 300 and 1000 m altitude in valleys and sheltered ravines ("barrancos") with remnants of (degraded) evergreen laurel

forest dominated by broad-leaved trees: *Laurus azorica* (Seub.) Franco, *Persea indica* (L.) Spreng., *Ocotea foetens* (Aiton) Berthel., *Apollonias barbujana* (Cav.) Bornm., *Ilex canariensis* Poir., *Ilex platyphylla* Webb and Berthel., and *Arbutus canariensis* Vieill. (Bramwell and Bramwell, 1974). *Asplenium aureum* usually grows in humus-rich soils, often together with *Adiantum capillus-veneris* L., *A. reniforme* L., *Anogramma leptophylla* (L.) Link, *Asplenium aethiopicum* (Burm.f) Bech., *A. hemionitis* L., *Cheilanthes pulchella* Bory ex Willd., *Davallia canariensis* (L.) Sm., *Polypodium cambricum* L. ssp. *macaronesicum* (A.E. Bobrov) Fraser-Jenk., and *Selaginella denticulata* (L.) Spring.

Asplenium ceterach and *A. octoploideum* were found in the natural pine forests ("Pinar") at 900–2000 m on Tenerife, and at 1200–1600 m on Gran Canaria. The octoploid was observed on La Palma at 700–1200 m. The open savannah-like vegetation is dominated by *Pinus canariensis* C. Sm. and a few shrubs, such as *Adenocarpus foliolosus* (Aiton) DC., *Cistus symphytifolius* Lam., *Daphne gnidium* L., *Micromeria* species, and *Rumex lunaria* L. (Bramwell and Bramwell, 1974). *Asplenium ceterach* and the octoploid grow in rock fissures, often together with *Monanthes laxiflora* (DC.) Bolle, *Aeonium* species, *Asplenium aethiopicum*, *A. trichomanes* L., *Anogramma leptophylla*, *Cheilanthes guanchica* Bolle, *C. pulchella*, *Cosentinia vellea* (Aiton) Tod., *Notholaena marantae* (L.) Desv. subsp. *subcordata* (Cav.) G. Kunkel, and *Polypodium cambricum* ssp. *macaronesicum*. Where *A. ceterach* and *A. octoploideum* grow together abundantly, we discovered their sterile hexaploid hybrid, *A.* ×*chasmophilum* Van den heede and Viane (Van den heede and Viane, 2002)

Discussion

In combination with morphological, cytological, and biogeographical data, isozyme markers can determine whether taxa are auto- or allopolyploid (Crawford, 1985; Haufler, 1985b; Bryan and Soltis, 1987; Weeden and Wendel, 1989; Crawford, 1990; Gastony, 1990; Pryer and Haufler, 1993). Electrophoretic analysis of isozymes is an ideal way to investigate the origin of allopolyploid taxa because parental loci are expressed as stable marker bands in the progeny (Haufler, 1985b; Werth *et al.*, 1985b; Gastony, 1986). The potential of isozyme data to clarify relationships in fern complexes is dependent upon the degree of differentiation among the ancestral genomes.

In the present study, four loci (*Aat, Skdh, Me*, and *Pgi-2*) showing a unique set of bands characterizing *A. aureum* and different from the banding patterns found in European *A. ceterach*, proved adequate to disentangle the "*Ceterach*" complex in the Canarian Archipelago.

All zymograms present in the Canarian "small tetraploid", were also observed in *A. ceterach*, and confirm that true *A. ceterach* is growing on the Canary Islands. Moreover, this suggests an occasional spore flow from Europe towards the Canaries. A flow in the opposite direction is less likely because the western islands are dominated by the northeast trade wind system. Conse-

quently, the Canarian *A. ceterach* population cannot be considered genetically isolated. No local zymotypes seem to have originated in this taxon in the Canary Islands, contrary to the situation on Cyprus, which is much older than the Canary Islands (Van den heede *et al.*, 2002). For example, the unique *Tpi-2* zymogram, present in all *A. ceterach* and *A. cyprium* specimens from Cyprus, suggests the local origin of the Cypriot taxa (Van den heede *et al.*, 2002).

All four loci (*Aat, Skdh, Me*, and *Pgi-2*) of the Canarian octoploid show monomorphic heterozygosity for a combination of the patterns seen in *A. ceterach* and *A. aureum*. Our allozyme data confirm the allo-octoploid nature of this species, which most probably originated by chromosome doubling in a tetraploid hybrid between *A. aureum* and *A. ceterach* (Viane and Reichstein, 1992). Theoretically, though less parsimoniously, the formation of this taxon could also happen directly via the union of unreduced (4x) gametes (on gametophytes resulting from unreduced spores) of both species.

All allozymes observed in the allo-octoploid were electrophoretically identical to those found in the parental tetraploids. However, in some octoploid samples from Gran Canaria and Tenerife, *Pgi-2* expressed a zymotype (corresponding to genotype *aaaadddd*) resulting from the combination of two undetected genotypes (*aaaa*) in *A. ceterach* and (*dddd*) in *A. aureum*. The occurrence of this putative "orphan" genotype may reflect incomplete sampling of the variation present in the tetraploids, or alternatively these genotypes may no longer be present in extant *A. ceterach* and *A. aureum* specimens.

The variation in the allo-octoploid seems to be related to the mono- or polymorphism (and its abundance) in the parental tetraploids. Thus, at the two loci (*Skdh* and *Me*) showing a single octoploid genotype, only one genotype was observed in each of the Canarian parents. At *Aat* two different genotypes were found for the Canarian *A. ceterach*, though only a single zymotype was detected for the octoploid. However, the *A. ceterach* genotype not detected in any octoploid, was found in only ca. 10% of the population. On the other hand, this may also be the result of limited sampling of the variation present in the octoploid.

The allo-octoploid species showed three different isozyme profiles at *Pgi-2*, a locus that is polymorphic in its tetraploid progenitors, indicating that the octoploid probably originated at least three times. According to Werth *et al.* (1985a) such patterns of variation in allopolyploids are almost certainly the result of repeated allopolyploidizations involving pairs of different genotypes. Thus, each of the octoploid zymotypes may have arisen from a separate hybridization event. The present observations, demonstrating multiple origins of allopolyploids, are similar to those of Werth *et al.* (1985a, b) for *Asplenium*, Soltis et al. (1987) for *Polystichum*, and Ranker et al. (1989) for *Hemionitis*. Recurrent origins of the allo-octoploid species implicate a repeated gene flow from tetraploids to octoploids, and mean a continued gain of genetic diversity by the allopolyploid.

Our electrophoretic data also provide evidence for the operation of tetrasomic inheritance in natural populations of autotetraploid (Rasbach *et*

al., 1987) *A. ceterach*. At *Skdh*, for which only two allozymes were observed, three types of heterozygotes were present: balanced heterozygotes (*ccdd*) and two types of unbalanced heterozygotes (*cccd, cddd*). The presence of these three types in tetraploid *A. ceterach* at *Skdh* is suggestive of the three possible classes of heterozygotes expected in an autotetraploid at a locus having two alleles (Weeden and Wendel 1989). Unbalanced staining activities indicate multiple doses of individual alleles. Tetrasomic inheritance with chromatidal segregation explains the arrays of homozygous, balanced heterozygous, and unbalanced heterozygous banding patterns observed in *A. ceterach* (Weeden and Wendel 1989). Tetrasomic inheritance implies that a chromosome can pair with any of its three homologous chromosomes (e.g., Soltis and Rieseberg, 1986; Weeden and Wendel, 1989; Crawford, 1990), and that there is apparently no strict preferential chromosome pairing. Consequently, the present isozyme analysis confirms the autotetraploid status of *A. ceterach*, which was cytologically proven by Rasbach et al. (1987). The fact that isozyme studies point to tetrasomic inheritance and that we found only bivalents during meiosis in autotetraploid *A. ceterach*, suggests that both processes are controlled by different (sets of) genes. Similar unbalanced patterns found in allotetraploid ferns have been explained also by segregating intralocus heterozygosity and fixed interlocus heterozygosity (Gastony, 1990).

We were able to prove, by isozyme and plastid DNA analysis, that in addition to *A. aureum* and the octoploid, true *A. ceterach* occurs on Gran Canaria and Tenerife. A combination of morphological and cytological analysis leads to correct determination, but even the exospore length alone allows reliable identification of the three Canarian species: *A. aureum* (32 ± 1.9 μm), *A. ceterach* (39 ± 2.6 μm), and the octoploid (44 ± 3.1 μm).

As mentioned in the introduction, Benl and Kunkel (1967) published *C. aureum* var. *parvifolium* without cytological investigation. Plants collected in 1967 by T. Reichstein and G. Kunkel were found to be octoploid, leading T. Reichstein and other European pteridologists to attribute octoploid status to *A. parvifolium* (including all small Canarian "*Ceterach*" specimens), but without having checked the holotype.

We repeatedly visited the type locality of *A. parvifolium*, the Pinar above Vilaflor (Tenerife), and found several taxa (see Appendix 3) growing together. As soon as we were convinced that two kinds of "small *Ceterach*" species were growing at the locus classicus (and on Gran Canaria), we decided to study the holotype (*Benl s.n.*, 26/12/1966, M) of *C. aureum* var. *parvifolium*. This holotype consists of one single plant. We studied its microcharacters (see also Table 4) and found a mean exospore length (38 ± 2.8 μm) and very few folds ("cuticular lines") in the scales characteristic for true *A. ceterach*. The values for the exospore and stomate length (38 ± 3.7 μm) prove that the holotype was not an octoploid, but a tetraploid plant! Consequently, *C. aureum* var. *parvifolium* Benl and G.Kunkel and *A. parvifolium* are synonyms of *A. ceterach*, and the octoploid had no correct name and was described as *A. octoploideum* (Van den heede and Viane, 2002). *Asplenium octoploideum* is morphologically intermediate between *A. aureum* and *A. ceterach*, from which

TABLE 4. Comparison of mean exospore length (LEXO) of various types to that of cytologically checked vouchers (see Table 2).

Taxon	Voucher, status, and herbarium	LEXO ± s.d. (types)	LEXO ± s.d. (cytol. checked)
A. aureum	*Broussonet s. n.*, iso-:P	31 ± 1.6 μm	32 ± 1.9 μm
A. ceterach	*Hort. Cliff. Aspl. 4*, lecto-: BM	39 ± 2.8 μm	39 ± 2.6 μm
A. parvifolium	*Benl s. n.*, holo-: M	38 ± 2.8 μm	
A. octoploideum	*CV 188*, holo-: GENT	42 ± 3.4 μm	44 ± 3.1 μm

it can be distinguished by its different mean exospore length (44 μm) and mean stomate length (52 μm), and its octoploid chromosome number $n = 144^{II}$ (Fig. 2B + B'). It is endemic to the Canarian Archipelago but presently confirmed only (cytology) for Gran Canaria, La Palma, and Tenerife, and to be expected on La Gomera and El Hierro. In addition to the holotype from Gran Canaria [lava field near Cueva Corcho, in fissures of volcanic rocks, 1350 m alt, 28th May 1997, leg. *Van den heede and Viane CV 188* (Holo-: GENT, iso-: personal herbarium of Viane and Van den heede)], the following collections (paratypes) were also made (for localities see Appendix 1, 2, and 3): *CV 171, CV 172, CV 173, CV 175, CV 176, CV 177, CV 179, CV 672A+B, CV 674, CV 686, CV 687, CV 695, CV 708, CV 709, CV 715, CV 716, CV 717, CV 718, CV 719A+B, CV 720, CV 721, CV 723, CV 724, CV 725, CV 726, CV 727, CV 729, CV 730A+B, CV 731, CV 732, CV 733, CV 734, CV 740, CV 741, CV 742, CV 743, CV 744, CV 745, CV 746, CV 747, CV 748, CV 749, CV 750, CV 751A+B, CV 752, CV 754A+B+C, WB 22/93.*

Many herbarium specimens still need to be inspected before the ranges of the "small Canarian *Ceterach*" taxa can be established. Literature references and information on herbarium labels are often unreliable, e.g., *Bornmüller 3094* (P), labeled as *C. officinarum* f. *typica* (*cellulis palearum non striatis*!) collected on Gran Canaria, has a mean exospore length of 44 ± 3.0 μm and numerous folds in the scale cells: it is without any doubt an octoploid.

As far as is known, the octoploid is endemic to the Canary Islands, but because Madeira could also harbor this species (climatologically and topographically), we studied 30 specimens from five Madeiran localities. However, all of them turned out to be hexaploid and were identified as *A. lolegnamense*. Both in the Madeiran and the Canarian Archipelago the northeast trade wind prevails during the year. This phenomenon may help to explain the restricted range of several Macaronesian taxa, because most propagules fall into the Atlantic Ocean. Even when spores occasionally reach the African continent, the Western Sahara and Mauritania offer no appropriate habitats for these ferns, because of their ultra dry climate. The derivation we hypothesize for the allo-octoploid species is presented in Fig. 9.

It is generally accepted (e.g., Burchard, 1929; Lems, 1960; Page, 1973; Bramwell and Bramwell, 1974; Page, 1977, 1979) that many of the Canarian ferns are endemic relicts of the Tertiary fern flora that existed in southern Europe during the Miocene and Pliocene. These ferns form an important part of the original vegetation of the Canary Islands, especially of the evergreen

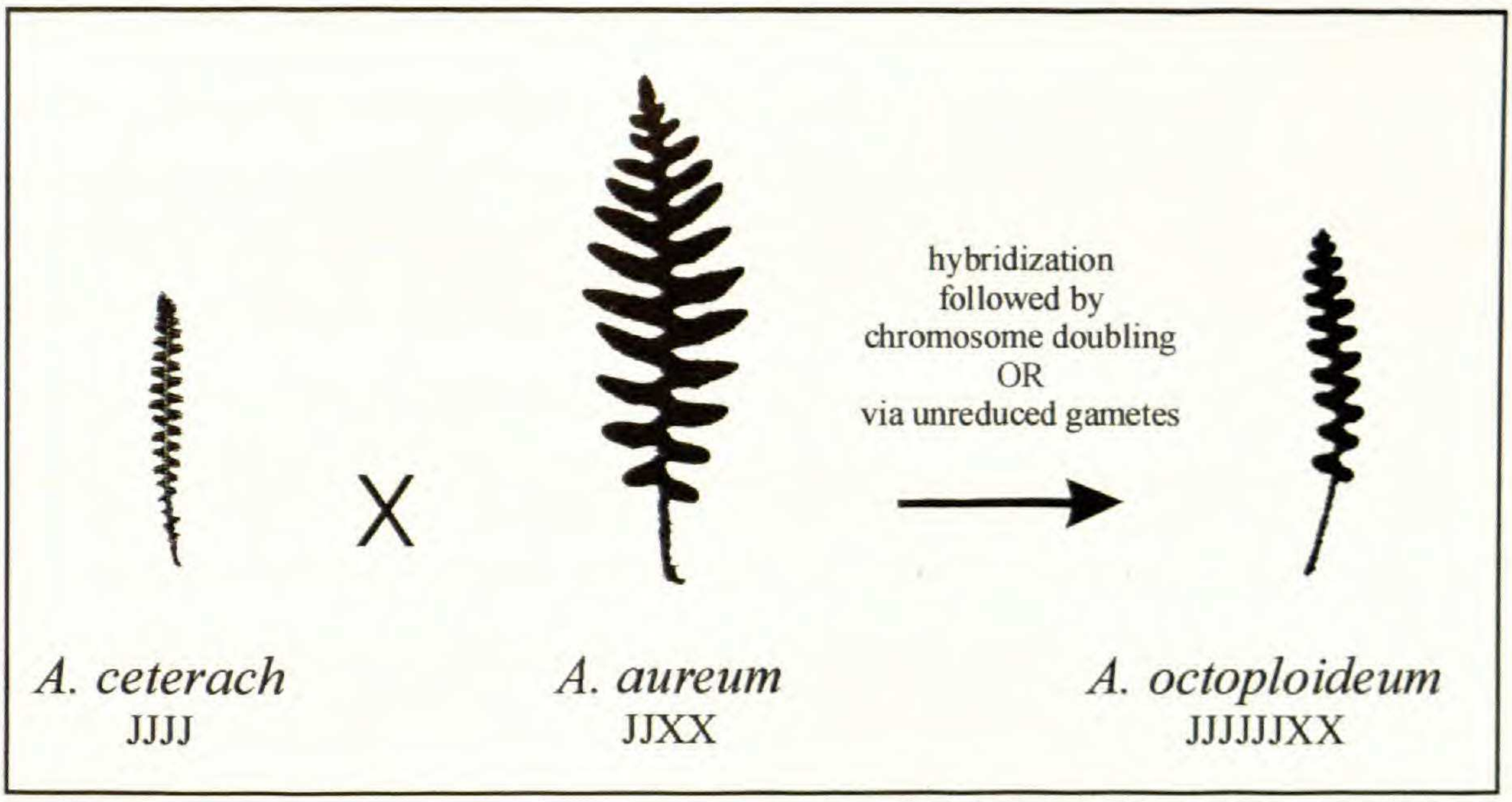

FIG. 9. Scheme of relationships explaining the origin of the Canarian *Asplenium octoploideum* based on (micro)morphological, cytological and molecular data. Each capital represents one set of 36 ancestral chromosomes. The JJ genome represents *A. javorkeanum*. Because molecular studies (Van den heede et al., 2003) suggest that *A. aureum* is an allotetraploid, involving *A. javorkeanum* (JJ) and "*A. semi-aureum*" (XX, unknown) as ancestors, its genome formula is given as JJXX.

forests (Page, 1977). Unfortunately, these habitats, if not totally destroyed today, are greatly endangered by modern tourism (building, water supply). Several mountain areas need further research, and new species and hybrids await description. Undoubtedly, this Tertiary (fern) flora forms an irreplaceable genetic resource that should be conserved.

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APPENDIX 1. Vouchers from Gran Canaria, with corresponding taxa, locality numbers, and chromosome numbers. CV is the abbreviation for Caroline Van de heede. The description of the localities and their number is given in Material and Methods. For samples with chromosomes counted, the number of bivalents is given. Counted specimens served as standards to determine the ploidy level (indicated by 4x and 8x) by flow cytometry. All specimens were used for isozyme analysis.

Voucher number	Taxon	Locality	Date of collection	Meiotic chromosome number (n), or ploidy
CV 157	*A. aureum*	1	25 May 1997	72^{II}
CV 158	*A. aureum*	1	25 May 1997	4x
CV 159	*A. aureum*	1	25 May 1997	4x
CV 160	*A. aureum*	1	25 May 1997	72^{II}
CV 161	*A. aureum*	1	25 May 1997	72^{II}
CV 162	*A. aureum*	1	25 May 1997	4x
CV 163	*A. aureum*	1	25 May 1997	4x
CV 164	*A. aureum*	1	25 May 1997	72^{II}
CV 165	*A. ceterach*	2	25 May 1997	72^{II}
CV 166	*A. ceterach*	2	25 May 1997	4x
CV 167	*A. ceterach*	2	25 May 1997	4x
CV 168	*A. ceterach*	2	25 May 1997	
CV 169	*A. ceterach*	2	25 May 1997	4x
CV 170a	*A. ceterach*	2	25 May 1997	4x
CV 170b	*A. ceterach*	2	25 May 1997	72^{II}
CV 171	*A. octoploideum*	2	25 May 1997	8x
CV 172	*A. octoploideum*	2	25 May 1997	8x
CV 173	*A. octoploideum*	3	26 May 1997	8x
CV 175	*A. octoploideum*	3	26 May 1997	8x
CV 176	*A. octoploideum*	3	26 May 1997	8x
CV 177	*A. octoploideum*	3	26 May 1997	8x
CV 179	*A. octoploideum*	3	26 May 1997	8x
CV 188	*A. octoploideum*	3	28 May 1997	144^{II}
CV 180	*A. aureum*	4	26 May 1997	4x
CV 181	*A. aureum*	4	26 May 1997	4x
CV 182	*A. aureum*	4	26 May 1997	

APPENDIX 2. Vouchers from La Palma, with corresponding taxa, locality numbers, and chromosome numbers. CV is the abbreviation for Caroline Van den heede. The description of the localities and their number is given in Material and Methods. For samples with chromosomes counted, the number of bivalents is given. Counted specimens served as standards to determine the ploidy level (indicated by 4x and 8x) by flow cytometry. All specimens were used for isozyme analysis.

Voucher number	Taxon	Locality	Date of collection	Meiotic chromosome number (n), or ploidy
CV 708	A. octoploideum	5	2 Apr. 1999	8x
CV 709	A. octoploideum	5	2 Apr. 1999	144^{II}
CV 711	*A. aureum*	6	3 Apr. 1999	4x
CV 712	*A. aureum*	6	3 Apr. 1999	4x
CV 713	*A. aureum*	6	3 Apr. 1999	
CV 715	*A. octoploideum*	7	4 Apr. 1999	8x
CV 716	*A. octoploideum*	7	4 Apr. 1999	8x
CV 717	*A. octoploideum*	8	4 Apr. 1999	8x
CV. 718	*A. octoploideum*	8	4 Apr. 1999	8x
CV 719A	*A. octoploideum*	8	4 Apr. 1999	8x
CV 719B	*A. octoploideum*	8	4 Apr. 1999	8x
CV 720	*A. octoploideum*	8	4 Apr. 1999	8x
CV 721	*A. octoploideum*	8	4 Apr. 1999	8x
CV 723	*A. octoploideum*	9	4 Apr. 1999	8x
CV 724	*A. octoploideum*	9	4 Apr. 1999	8x
CV 725	*A. octoploideum*	10	4 Apr. 1999	8x
CV 726	*A. octoploideum*	10	4 Apr. 1999	8x
CV 727	*A. octoploideum*	10	4 Apr. 1999	8x
CV 729	*A. octoploideum*	11	5 Apr. 1999	8x
CV 730A	*A. octoploideum*	11	5 Apr. 1999	8x
CV 730B	*A. octoploideum*	11	5 Apr. 1999	8x
CV 731	*A. octoploideum*	12	5 Apr. 1999	144^{II}
CV 732	*A. octoploideum*	12	5 Apr. 1999	8x
CV 733	*A. octoploideum*	12	5 Apr. 1999	8x
CV 734	*A. octoploideum*	12	5 Apr. 1999	8x
CV 740	*A. octoploideum*	13	9 Apr. 1999	8x
CV 741	*A. octoploideum*	13	9 Apr. 1999	8x
CV 742	*A. octoploideum*	13	9 Apr. 1999	8x
CV 743	*A. octoploideum*	13	9 Apr. 1999	8x
CV 744	*A. octoploideum*	13	9 Apr. 1999	8x
CV 745	*A. octoploideum*	13	9 Apr. 1999	8x
CV 746	*A. octoploideum*	13	9 Apr. 1999	8x
CV 747	*A. octoploideum*	13	9 Apr. 1999	8x
CV 748	*A. octoploideum*	13	9 Apr. 1999	8x
CV 749	*A. octoploideum*	13	9 Apr. 1999	8x
CV 750	*A. octoploideum*	13	9 Apr. 1999	8x

APPENDIX 3. Vouchers from Tenerife, with corresponding taxa, locality numbers, and chromosome numbers. CV, RV, and WB are abbreviations for Caroline Van den heede, Ronald Viane, and Wilfried Bennert. The description of the localities and their number is given in Material and Methods. For samples with chromosome counted, the number of bivalents is given. Counted specimens served as standards to determine the ploidy level (indicated by 4x and 8x) by flow cytometry. All specimens were used for isozyme analysis.

Voucher number	Taxon	Locality	Date of collection	n or ploidy
RV 6135	*A. ceterach*	14	7 May 1995	72^{II}
CV 183	*A. ceterach*	14	27 May 1997	72^{II}
CV 184	*A. ceterach*	14	27 May 1997	72^{II}
CV 185	*A. ceterach*	14	27 May 1997	4x
CV 186	*A. ceterach*	14	27 May 1997	72^{II}
CV 187a	*A. ceterach*	14	27 May 1997	72^{II}
CV 187b	*A. ceterach*	14	27 May 1997	4x
CV 187c	*A. ceterach*	14	27 May 1997	4x
CV 663B	*A. ceterach*	14	11 Jan. 1999	4x
CV 665	*A. aureum*	15	12 Jan. 1999	4x
CV 666	*A. aureum*	15	12 Jan. 1999	4x
CV 667	*A. aureum*	16	12 Jan. 1999	
CV 668	*A. aureum*	16	12 Jan. 1999	
CV 669	*A. aureum*	16	12 Jan. 1999	4x
CV 670	*A. aureum*	16	12 Jan. 1999	72^{II}
CV 671	*A. aureum*	16	12 Jan. 1999	4x
CV 672A	*A. octoploideum*	17	13 Jan. 1999	8x
CV 672B	*A. octoploideum*	17	13 Jan. 1999	8x
CV 674	*A. octoploideum*	17	13 Jan. 1999	8x
CV 675	*A. aureum*	18	14 Jan. 1999	4x
CV 676	*A. aureum*	18	14 Jan. 1999	4x
CV 677	*A. aureum*	18	14 Jan. 1999	4x
CV 678	*A. aureum*	18	14 Jan. 1999	4x
CV 683	*A. ceterach*	19	15 Jan. 1999	4x
CV 684	*A. ceterach*	19	15 Jan. 1999	4x
CV 686	*A. octoploideum*	19	15 Jan. 1999	8x
CV 687	*A. octoploideum*	19	15 Jan. 1999	8x
CV 695	*A. octoploideum*	19	15 Jan. 1999	8x
CV 696	*A. ceterach*	19	15 Jan. 1999	4x
CV 701	*A. ceterach*	20	15 Jan. 1999	4x
CV 702A	*A. ceterach*	20	15 Jan. 1999	4x
CV 702B	*A. ceterach*	20	15 Jan. 1999	4x
CV 704	*A. ceterach*	20	15 Jan. 1999	4x
CV 705	*A. aureum*	21	16 Jan. 1999	4x
CV 706	*A. aureum*	21	16 Jan. 1999	4x
CV 707	*A. aureum*	21	16 Jan. 1999	4x
CV 751A	*A. octoploideum*	22	10 Apr. 1999	8x
CV 751B	*A. octoploideum*	22	10 Apr. 1999	
CV 752	*A. octoploideum*	22	10 Apr. 1999	8x
CV 754A	*A. octoploideum*	23	10 Apr. 1999	8x
CV 754B	*A. octoploideum*	23	10 Apr. 1999	8x
CV 754C	*A. octoploideum*	23	10 Apr. 1999	8x
WB 22/93	*A. octoploideum*	24	15 Apr. 1993	144^{II}

APPENDIX 4. Alphabetical list of material for comparison used in this study. CV, RV, TR, and WB are abbreviations of C. Van den heede, R. Viane, T. Reichstein, and W. Bennert. Vouchers are deposited in GENT and in our personal herbarium at Ghent University. Additional information about localities is available from the first and the last author (lienvdheede@hotmail.com; ronnie.viane@UGent.be). Voucher information about 108 Cypriot samples is given in Van den heede et al. (2002).

Asplenium ceterach:	
CV25b+c	Slovenia, Kal
CV25b, CV30a+b, CV31	Croatia, Roč
CV36a+b, CV37	Croatia, Bassania
CV38b	Slovenia, Korte
CV41,CV42b, CV44,CV48, CV49b	Italy, Valle della Marossa
CV64, CV65, CV66, CV67, CV494	Italy, Termine di Roverano
CV225	Cyprus, Troodos Mts., Chandria
CV275a+b, CV276, CV277	Belgium, Marcourt
CV278, CV279	Italy, Cannero Riviera
CV281a+b, CV282, CV283, CV285, CV286	United Kingdom, Wales, Snowdonia
CV429, CV430, CV431	Crotia, Losinj
CV445, CV448, CV449	Italy, Berceto
CV450, CV451	Italy, Boio
CV657, CV658, CV659	Spain, Torrelodones
CV767, CV768, CV769, CV770, CV771	Spain, Alava
CV773	France, Coulgens
CV774, CV775, CV776	France, Paulmy
RV5900	France, NNE of Montpellier, La Pene
WB1b+e/97	Turkey, Karaoba
WB10d/97	Turkey, Manisa
WB5c/97	Turkey, Okçular
WB12c+d/97	Turkey, Mugla
Asplenium cyprium:	
CV213	Cyprus, Troodos Mts., Tsakistra-Vroiska road
CV249	Cyprus, Kyrenia Mts., Kyrenia-Kythrea road
Asplenium dalhousiae	
CV318	Ethiopia, Harerge Province, Asbe Teferi
TR7634	Pakistan, Swat Province, Ambela
Asplenium javorkeanum:	
CV3, CV4, CV5	Italy, Stupizza
CV7a+b+c, CV8a+b+c	Slovenia, 1 km E of Bača towards Podbrdo
CV10, CV11, CV12, CV14, CV412, CV414	Slovenia, Bača-valley, Kneža-Klavže road
CV20a+b+c+d, CV21a+b+c+d+e	Slovenia, Matavun
CV81, CV82a+b, CV83, CV84, CV85a+b	Italy, Arni
CV86, CV87, CV88	Italy, Monte Freddone, 730m
CV89, CV90, CV91, CV92, CV93	Italy, Monte Freddone, 1320m
CV94a+b, CV95	Italy, Monte Freddone, 970m
CV404, CV405	Slovenia, Bovec-Kobarid road
CV410	Slovenia, Ljubinj
CV480	Italy, N slope of Pania Secca
CV483, CV484	Italy, Fosso di Antona
CV504, CV506	Italy, E slope of Monte Corchia
Asplenium lolegnamense:	
CV985	Madeira, SW slope of Pico Ruivo
CV993	Madeira, N of Serra de Agua